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Persistence and diversity of Theileria parva in East Coast fever vaccinated and unvaccinated cattle

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PERSISTENCE AND DIVERSITY OF THEILERIA PARVA IN EAST COAST FEVER VACCINATED AND UNVACCINATED CATTLE

Emelesiana Cyprian Magulu

A dissertation submitted in partial fulfillment of the requirements for the degree of Master's in Life Sciences of the Nelson Mandela African Institution of Science and Technology

Arusha, Tanzania

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ABSTRACT

Infection and Treatment Method (ITM) has been practiced in Tanzania for over 20 years now as a prevention method against East Coast Fever (ECF). However, there is less information regarding the persistence of the *Theileria parva* parasites after a long-time lapse after vaccination. Likewise, the implication of the potential exchange of genetic information is largely unknown as regards ECF vaccination outcomes. A total number of 336 cattle samples were collected from Loiborsoit, Emboret, Esilalei, Manyara ranch, Mswakini, Tanga Mruazi farm and Tanga Leila farm in March of 2018. Samples were then screened for *Theileria parva* using nested PCR and the overall prevalence of carrier state was 34.5%, with a significant higher prevalence among the vaccinated cattle group 43% (103/239) compared to the unvaccinated group 13.4% (13/97) ($p<0.000$). Similarly, higher prevalence emerged among the cattle grazing close to the wildlife 38.2% (107/280) compared to the ones grazing far from wildlife 16.1% (9/56) $p<0.001$. The carrier state persisted up to 132 months post vaccination. Minisatellite 7 (MS 7), microsatellite 2 and 5 (ms 2, ms 5) markers were used to characterize genetic diversity. Whereby parasite diversity across cattle groups was determined by the mean number of alleles, and expected heterozygosity. Manyara ranch had the highest parasite diversity with all the markers. This study concludes that vaccination against ECF and the wildlife interface has an influence on the diversity of *Theileria parva* parasites, as the highest number of alleles and parasite diversity were shown in the vaccinated cattle and the ones in close proximity to wildlife interface.

Key words: East Coast Fever, Infection and Treatment Method, Northern Tanzania, *Theileria parva*

DECLARATION

I, Emelesiana Magulu do hereby declare to the Senate of the Nelson Mandela Institution of Science and Technology that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

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Emelesiana Cyprian Magulu Date

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by the Nelson Mandela African Institution of Science and Technology a dissertation entitled "Persistence and diversity of *Theileria parva* in East Coast Fever vaccinated and unvaccinated cattle in northern Tanzania", in partial fulfillment of the requirements for the degree of Master's in Life Sciences of the Nelson Mandela African Institution of Science and Technology, Arusha, Tanzania.

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Dr. Gabriel Shirima Date
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LIST OF ABREVIATION AND SYMBOLS

AMOVA	Analysis of molecular variance
Bp	Base pair
$CD8^+$	Cluster of differentiation
CTL	Cytotoxic T Lymphocytes
℃	Degrees centigrade
%	Percentage
&	And
DF	Degree of freedom
EDTA	Ethylene diaminetetra acetic acid
et al	And others
gDNA	Genomic DNA
ITM	Infection and treatment method
ITS	Internal transcribed spacer
Kbp	Kilo base pair
LAMP	Loop mediated isothermal amplification
LSUrRNA	Large subunit ribosomal RNA
MAbs	Monoclonal antibodies
Mbp	Mega base pairs
MC	Muguga cocktail
MHC	Major histocompatibility complex
ms	Microsatellite
MS	Minisatellite
nPCR	Nested PCR
PIM	Polymorphic immunodominant
Pmole	Picomole
PCA	Real time PCR
qPCR	Principal component analysis
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
SSUrRNA	Small subunit ribosomal RNA
SS	Sum of squared differences

VNTR	Variable number tandem repeat

CHAPTER ONE

INTRODUCTION

1.1 Background information

Theileria parva (*T. parva*) is a tick-borne protozoan parasite belonging to the phylum Apicomplexa and causes a severe disease in cattle known as East Coast Fever (ECF) (Norval, Perry, & Young, 1992). This disease is endemic in eleven countries in eastern and central Africa where it results into serious economic losses to the livestock industry (Hayashida *et al*., 2012). The natural host of *T. parva,* African buffalo (*Syncerus caffer*) does not experience any clinical signs of the disease. *Theileria parva* is a tick borne parasite, transmitted by the tick vector, *Rhipicephalus appendiculatus*, which feeds on the infected animal and spreads the infection (Olds, Mason, & Scoles, 2018). The high likely breeds to suffer high morbidity and mortality cattle are (*Bos taurus*) and their crosses which are mostly used for milk production (Nene & Morrison, 2016).

The severity of ECF depends on the parasite dose and there are also differences in magnitude of infection in each cattle (Cunningham *et al*., 1974; Dolan, 1986; Nene *et al*., 2016). Poor small-holder farmers are the ones experiencing great impacts resulted from this disease as they often do not have enough resources and access to control measures. The mortality may reach 100%, in exotic cattle however for indigenous cattle (*Bos indicus*) breeds there is a development of resistance to the disease right after primary natural infection and recovery. Cattle that recover from ECF do not get rid of the infection, rather they remain as carriers of the parasite and a source of infections to ticks (Kariuki *et al*., 1995). This event plays a part in endemic stability of the disease in indigenous cattle especially in areas where there is continual challenges of the parasite (Norval *et al*., 1992)

Theileria parva infects cattle and the African buffalo (*Syncerus caffer*), the parasite is known to progress with the buffalo in eastern Africa (Uilenberg, 1981). Despite this the infected buffalo do not normally display any clinical signs of the disease, whereas in cattle the parasite causes a severe fatal lympho proliferative disorder called ECF (Sitt *et al*., 2015). Theiler (1912) was the first to recognize *Theileria parva* as the causative agent of ECF in South Africa. He differentiated ECF from Red water caused by Babesia specie, and recognized the principal tick vector that transmits *T. parva* as *Rhipicephalus appendiculatus* (Nene *et al*., 2016). The disease was associated with cattle imported from

East Africa, and caused high levels of morbidity and mortality (Norval *et al*., 1992). South Africa eradicated the disease by slaughtering of infected cattle, a control of cattle movement and fencing to avoid buffalo to game parks (Nene *et al*., 2016). There are still occasional outbreaks of buffalo-derived *T. parva* disease, but appear to be self-limiting in nature (Mbizeni *et al*., 2013; Thompson, Oosthuizen, Troskie, & Penzhorn, 2008). However, in other countries it has not been possible to implement such measures which resulted to ECF being an endemic acute and lethal disease in 12 countries in eastern, central and southern Africa, including Burundi, Democratic Republic of Congo, Kenya, Malawi, Mozambique, Rwanda, South Sudan, Tanzania, Uganda, Zambia and Zimbabwe (Malak *et al*., 2012; Norval *et al*., 1992), and in the Comoro Islands (De Deken *et al*., 2007).

On a regional basis ECF kills approximately 1 million cattle/year with annual economic losses of approximately USD300 (Malak *et al*., 2012; McLeod *et al*., 1999). The impacts are likely to increase with time. In Tanzania, the disease accounts for the 43.7% of annual mortality of livestock and was estimated to have an overall annual cost of 43 million USD (McLeod & Kristjanson, 1999). East coast fever is responsible for annual mortality rates of 40– 80% in unvaccinated Zebu (*Bos indicus*) calves (Di Giulio, Lynen, Morzaria, Oura, & Bishop, 2009; Homewood, Trench, Randall, Lynen, & Bishop 2006). On top of that ECF accounts for up to 70% of deaths in 6 – 8 months old calves in pastoral herds which renders serious threats to smallholder dairy farmers for whom the death of a single dairy cow can cause a measurable economic setback (Kazungu, Mwega, Kimera, & Gwakisa, 2015). East coast fever contributes to major constraint to improved productivity of cattle.

Initially ECF control relied on intensive application of acaricides (Norval *et al*., 1992), however, this practice has been abandoned with time due to several factors mostly development of resistance by ticks, other factors include financial constraints, disruption of endemic stability and environmental impact (Di Giulio *et al*., 2009). An efficient way to succumb this disease is immunization of cattle by the Infection and Treatment Method (ITM) (Oura, Bishop, Wampande, Lubega, & Tait, 2004). The method works by the injection of known strain(s) of *T. parva* followed by a simultaneous administration of an antibiotic (30% tetracycline), which leads to an attenuate infection resulting in a long-lasting immune response (Di Giulio *et al*., 2009; McKeever, 2009). In order to offer broad protection against most field isolates the vaccine is made by the combination of different strains (McKeever, 2009; Uilenberg, 1999), like with the trivalent vaccine known as the Muguga Cocktail (MC)

comprising of Muguga, Kiambu 5 and Serengeti-transformed stocks (McKeever, 2007; Ruheta, 1999). Muguga Cocktail is a live vaccine, therefore it requires cold chain system for its maintainance and eventually establishes a continous carrier status (Di Giulio *et al*., 2009; Uilenberg, 1999). There are worries that this may lead to introduction of specific vaccine parasite strains into the field, this would result in possible genetic recombinations with local circulating parasite strains (Oura *et al*., 2007) or the development of the disease in areas where it never existed before (McKeever, 2007; Uilenberg, 1999). There is therefore a need to address these concerns especially in Tanzania where ITM has been practiced for over 20 years.

1.2 Problem statement

Control of ECF relying on prevention of tick infestation is not only expensive but difficult to sustain as it requires continuous application of acaricides. The frequent use of acaricides presents threats to the environment. Drugs can be used as an alternative but they are only efficient when animals are treated during early stages of the disease and they are very costful. Due to the shortcomings of these control measures and the fatal nature of the disease, there is a demand for effective vaccines to provide a sustainable means of controlling the disease. Vaccination against ECF is based on an Infection and Treatment Method (ITM) that involves inoculation of live sporozoite-stage parasites and simultaneous treatment with long-acting tetracycline (McKeever, 2007). The vaccine, also called the Muguga cocktail, consists of the three strains of *T. parva*, Muguga, Serengeti-transformed and Kiambu 5. However, there are still questions regarding the antigenic composition of the vaccine and its effectiveness in obtaining strong immunity in different geographical and ecological locations. Importantly, there are worries that the Muguga cocktail vaccine may introduce parasites with a new genetic background into local parasite populations. Since ticks do not choose on which cattle to feed on this may result in into recombination and existence of more diverse strains of *T. parva*. In spite of such worries, the infection and treatment method has found wide adoption in pastoral areas of northern Tanzania in the last 20 years. Infection and Treatment Method (ITM) has reduced calf mortality from 80 to less than two per cent, this enabled the cattle herders being able to sell more animals and increase their income. Despite the high cost of the vaccine up to US$10 per animal (Di Giulio *et al*., 2009), the ITM remains to be the most efficient cost-effective ECF control option available to farmers and livestock keepers in Tanzania (Martins, Di Giulio, Lynen, Peters, & Rushton, 2010).

Vaccinated cattle and the ones that recover from natural infection develop immunity to re-infection with similar strains which contributes to endemic stability of the disease in indigenous cattle in areas with continuous transmission of the parasites. Apart from being immune those cattle do not usually eliminate the infection; and they remain as carriers of the parasite which acts as a source of infections to ticks. A study done in northern Tanzania by Kazungu, Mwega, Kimera and Gwakisa (2015) on ECF-vaccinated herds demonstrated that continuous natural tick challenge provides an incremental effect on acquired immunity. Furthermore, ticks continuously feeding on the carrier animals they transmit infections to non-infected cattle which deploys some of the vaccine parasites to unvaccinated cattle leading into high seroprevalence (Kazungu *et al*., 2015). Although ITM has been shown to have a significant impact in reducing ECF incidences in pastoral herds, there are still several questions which require focused research.

1.3 Rationale of the study

East Coast Fever is a significant economic burden for the small-holder pastoral livestock keepers in Tanzania. Vaccinated and non-vaccinated cattle populations in these areas co-graze using same pastures and with close proximity to the wildlife interface. Given that *Theileria* parasites could recombine between divergent strains during the sexual stage in ticks, 'vaccine-derived' and 'local' strains could exchange genetic information, resulting in parasites with genetic mosaics and diversity. The implication of the potential exchange of genetic information is largely unknown as regards ECF vaccination outcomes. It is important therefore to determine the persistence and diversity of the ECF vaccine (Muguga) components in relation to vaccination status and proximity to wildlife interface areas and to distinguish cattle potentially responding to the ITM vaccine strains alone, from those whose immunity is due to natural infection. According to Oura, Bishop, Lubega, and Tait, (2004) sustained use of live vaccination is likely to modify transmission dynamics and parasite population genetics. Livestock keepers in northern Tanzania have been vaccinating their calves now for more than fifteen years. There is therefore a need to understand the long-term impact of the potential genetic recombination in ticks and interface with wildlife on outcomes of ECF vaccination in cattle populations vaccinated over different time points (1-15 years lapse). The long-term impact of this project is to assist poor smallholder and pastoralist livestock keepers in Tanzania through research that will improve the control of ECF through vaccination.

1.4 Objectives

1.4.1 General objective

To enhance understanding of the long term impact of ITM in vaccinated and unvaccinated cattle.

1.4.2 Specific objectives

(i) To determine the prevalence of *T. parva* carrier state in ECF vaccinated and unvaccinated cattle

(ii) To determine the persistence and diversity of *T. parva* in vaccinated and unvaccinated cattle.

1.5 Research questions

(i) What is the prevalence and persistence of the different ECF vaccine (Muguga coctail) components (Muguga, Kiambu 5 and Serengeti-transformed strains) in relation to vaccination status, proximity to wildlife interface areas and duration since vaccination (1-15 years)?

(ii) Are ECF vaccine strains detectable in the unvaccinated co grazing cattle?

(iii) Is diversity of ECF vaccine strains modified due to presence of local *T. parva* strains?

1.6 Significance of the research

Outputs of this study will help to better understand long term impacts of the infection and treatment method for ECF control. Key expected outputs include the improvement of ECF vaccination regimes through, evidence for establishment of the three Muguga vaccine strains (Muguga, Kiambu 5 and Serengeti-transformed strains) in cattle vaccinated in the last 1-15 years. Such information will allow improvement of vaccine delivery in pastoral herds. Since available literature shows that, of the 3 strains present in the Muguga cocktail, only the Kiambu 5 stock establishes a long-term carrier state (up to 4 years) (Oura *et al*., 2007), this study will take advantage of the long term deployment of ECF vaccination for more than 15 years in Maasai communities of northern Tanzania to provide evidence on persistence of the three vaccine stocks (Kiambu 5, Muguga and Serengeti-transformed) under natural field conditions. Proof on the vaccine strains transmission to unvaccinated co grazing cattle and if the long-term application of ITM contributes to the diversity of *T. parva* vaccine strains.

1.7 Delineanation of the study

Infection and treatment method has been practiced as a method to control East Coast Fever disease among pastoral communities for more than 20 years. The method uses the vaccine known as Muguga Cocktail which consist of three different parasite strains of *T. parva* known as Muguga, Kiambu 5 and Serengeti transformed. Previous studies have been done on the vaccine and its persistence after administration however, the available information is limited for up to 4 years after vaccination.

This dissertation deals with the persistence of the Muguga Cocktail vaccine after 15 years of administering it among the pastoral communities in northern Tanzania, this stand out as the available literature depicts this scenario for only up to four years and in different settings as the one used in this study. The dissertation looks into the genetic diversity of the parasites given the common practice of co grazing of vaccinated and unvaccinated cattle also sharing of pasture with the buffalo which is the main reservoir of the parasites.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Theileria Parva* and East Coast Fever (ECF)

2.1.1 Morphology of *T. parva*

Theileria parva is a tick-borne parasitic protozoan with a small genome of 10-12 Mbp. The haploid *T. parva* nuclear genome is 8.3×10ˆ6 base pairs (Mbp) in length. The species has four chromosomes and a plastome which contains the genes for the apicoplast, consists of one extremely A+T-rich region (>97%) about 3kbp in length that maybe the centromere. The *T. parva* nuclear genome contains about 4035 protein-encoding genes, which exhibits higher gene density a greater proportion of genes with introns, and shorter intergenic regions (Gardner *et al*., 2005).

2.2 Life cycle of *T. parva*

The vector that transmits *T. parva* known as *Rhipicephalus appendiculatus* is a three stage host tick. Sporozoites are produced in the salivary gland of the ticks and when feeding in an animal are the ones being transmitted, this occurs within 48 to 72 hours of being attached to the host. The sporozoites once inside the host's lymphocytes they develop into macroschizonts and then divide into two cells each containing schizonts, schizonts multiply and develop into merozoites/ microschizonts which invades the red blood cells and becomes piroplasms. Ticks ingest the red blood cells with the piroplasm and the sexual stage takes place inside the tick gut which results into formation of motile stage of *T. parva* which moves to the salivary glands of the ticks for the whole cycle to take place again.

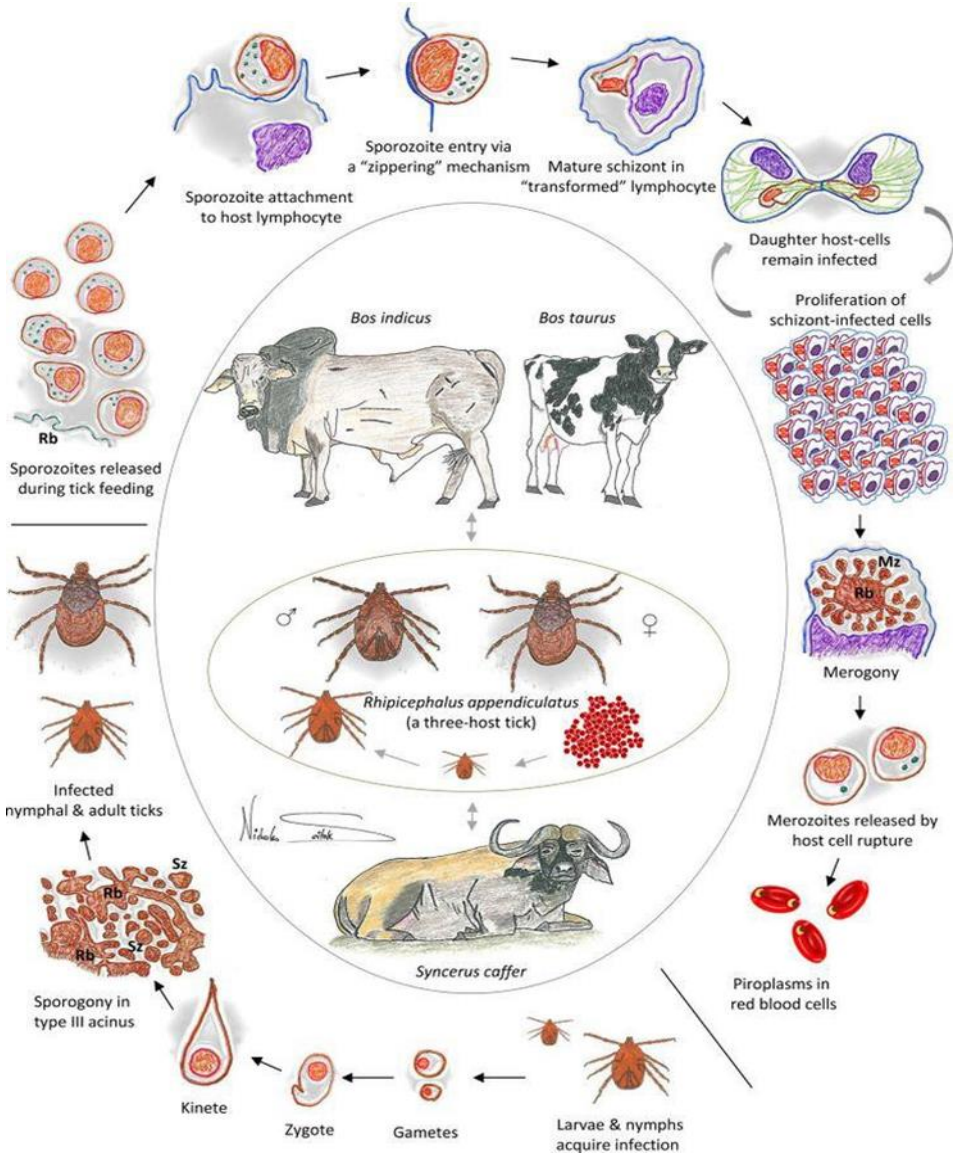

Figure 1: Life cycle of *T. parva* (Fawcett *et al*., 1982a; Norval *et al*., 1992; von Schubert *et al*., 2010).

2.3 Characterization of *T. parva*

The *T. parva* genes coding for antigen proteins (PIM, p67, p104, p150), ribosomal rRNA gene [Internal Transcribed Spacer (ITS), small subunit rRNA (SSUrRNA) and Large Subunit rRNA (LSUrRNA)] sequences have been analyzed in search for discriminatory differences between *T. parva* isolates (Baylis, Allsopp, Hall, & Carrington, 1993; Toye, Nyanjui, Goddeeris, & Musoke, 1996). The surface proteins PIM, p104, p67 and p150 can induce sporozoite-neutralizing antibodies and can also be used in discriminatory assays for *T. parva* isolates (Shapiro *et al*., 1987; Skilton *et al*., 1998). These surface proteins are

usually encoded by single copy genes and contain a polymorphic central region of amino acid sequences flanked by a conserved amino and carboxyl terminus (Skilton *et al*., 1998; Toye *et al*., 1995) whereas PIM and p150 are expressed in both sporozoite and schizont stages of the parasite, p67 and p104 are only expressed in the sporozoite stage (Katende *et al*., 1998; Shapiro *et al*., 1987).

Size polymorphisms displayed by *T. parva* antigen genes, PIM, p104, p150 and p67, have been used to develop several molecular tools for characterization of *T. parva* stocks, exploiting the variable regions of these genes (Bishop *et al*., 2001; Geysen, Bazarusanga, Brandt, & Dolan, 2004). Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) analysis using these antigen genes demonstrated polymorphism in field stocks of *T. parva* in Kenya, although the majority of field stocks isolated from two regions of Zambia were relatively homogeneous (Geysen, Bishop, Skilton, Dolan, & Morzaria, 1999). A semi-nested PCR is preferred for analysis of these genes to increase sensitivity since they are single copy genes.

2.3.1 Characterization of *T. parva* using mini- and micro-satellite markers

Minisatellite and microsatellite sequences show high levels of variation and therefore provide excellent tools for both the genotyping and population genetic analysis of parasites. Oura *et al*. (2003) used a panel of microsatellite and minisatellite markers to characterize field isolates of *T. parva.* A panel of 11 polymorphic microsatellites and 49 polymorphic minisatellites of the *T. parva* haemoprotozoan parasite were identified (Oura *et al*., 2003). The PCR *products* were run on high resolution spreadex gels on which the alleles were identified and sized. The sequences of the mini- and microsatellites were found to be distributed across the four parasite chromosomes with 16 on chromosome 1, 12 on chromosome 2, 14 on chromosome 3 and 18 on chromosome 4. The primers from the 60 sequences were also proved to be specific for *T. parva*.

When tissue culture isolates of *T. parva* isolated from cattle in widely separated African regions were characterized, the numbers of alleles per locus ranged from three to eight. This indicates a high level of diversity between these geographically distinct isolates. Analysis of isolates from cattle on a single farm identified a range of one to four alleles per locus which indicates a high level of diversity in a single population of *T. parva*. Cluster analysis of multilocus genotypes revealed that genetic similarity between isolates was not obviously related to their geographical origin (Oura *et al*., 2003). Mini- and microsatellite

sequences are frequently located in non coding regions, hence believed to be representative of population history (Odongo *et al*., 2006; Oura *et al*., 2003). Mini and microsatellites have been successfully used to determine population diversity of *T. parva* in different studies (Rukambile *et al*., 2016). Mini and microsatellite markers were used to characterize the three strains which are present as components of the Muguga *T. parva* vaccine cocktail, to determine the ability of each component to induce carrier state (Oura *et al*., 2004) and the possibility of being transmitted from vaccinated animals to other susceptible animals (Oura *et al*., 2007). Five mini and microsatellite markers were used to study diversity of the three stocks which are the components of the Muguga cocktail vaccine whereby 14 different genotypes were revealed (Patel *et al*., 2011). Furthermore, 9 minisatellites were used to study population genetics and substructure of *T. parva* population in the samples collected from two districts in Zambia with success (Muleya *et al*., 2012). However, the use of microsatellite markers is limited to parasite clones and cannot be directly used to characterize field samples, which usually contain complex mixtures of multiple *T. parva* strains.

2.3.2 Characterization of *T. parva* using antigen genes

(i) The *T. parva* p104 antigen gene

To characterize *T. parva* parasites occurring in buffalo (*Syncerus caffer*) in South Africa, the gene coding for the p104 antigen was selected for PCR-RFLP analysis. There is limited polymorphism in the p104 gene especially amongst cattle-type alleles. This allows distinction of buffalo-type from cattle-type alleles (Geysen *et al*., 1999). Skilton, Bishop, Katende, Mwaura and Morzaria (2002) identified four p104 alleles representing p104 amino acid sequences obtained from different *T. parva* stocks of cattle and buffalo in East Africa. Allele 1 (accession number: M2954) represents the *T. parva* Muguga p104 amino acid sequence (Iams *et al*., 1990); allele 2 (accession number: AY034069) is found in the Marikebuni and Uganda *T. parva* stocks and alleles 3 (accession number: AY034070) and 4 (accession number: AY034071) represent the *T. parva* Boleni and 7014 p104 amino acid sequences, respectively. Muguga, Marikebuni, Uganda and Boleni are cattle-derived *T. parva* stocks whereas 7014 is a buffalo-derived *T. parva* stock. The Polymorphic Immunodominant Molecule (PIM) and p104 profiles from buffalo-derived *T. parva* stocks are more polymorphic than those from cattle-derived stocks (Geysen *et al*., 1999).

2.4 Methods for characterization of *T. parva* diversity

2.4.1 Molecular techniques

(i) Conventional p104 PCR

This is a *T. parva* species-specific assay based on primers derived from conserved region of a 104-KDa antigen gene (p104) of *T. parva*. The assay was validated and showed a detection threshold (sensitivity) of 2 parasites/µl of infected blood (Skilton, Bishop, Katende, Mwaura, & Morzaria, 2002). However, just like the Reverse Line Blot technique, this assay is time-consuming due to the hybridization step.

(ii) Nested PCR for the p104 gene

Nested PCR involves use of two pairs of PCR primers for a single locus. The primers used in the first round of amplification are either both replaced (nested PCR) or only one is replaced (semi- nested PCR) for the second and subsequent cycles of amplification. The first pair amplifies the locus as in any conventional PCR experiment. The second pair of primers (nested primers) binds within the first PCR product and produces a second PCR product that is shorter than the first one. This strategy ensures that if the wrong locus were amplified during the first round PCR, the probability is very low that it would also be amplified a second time by a second pair of primers. Recent detection of *T. parva* infections has employed semi-nested PCR-RFLP assays based on the *T. parva* p104 and 18S rRNA genes (Bazarusanga, Vercruysse, Marcotty, & Geysen, 2007; De Deken *et al*., 2007). However, since p104 is a single copy gene, semi-nested PCR is less sensitive and not ideal for detection of *T. parva* infections in carrier animals. Oura *et al*. (2004) described a nested p104 PCR for detection of *T. parva* DNA in peripheral blood. The assay is based on amplification of a 277 bp internal fragment of the p104 gene which is both *T. parva*-specific and widely conserved within *T. parva* stocks (Bishop, Musoke, Morzaria, Gardner, & Nene, 2004; Skilton *et al*., 2002). The test exhibits enhanced sensitivity for detection of low levels of *T. parva* piroplasm infections in bovine carrier animals. Interpretation of the PCR result is based on visualization of a band on agarose gel. This study therefore employed nested PCR for the p104 gene for detection of *T. parva* in samples.

2.5 Control of *T. parva*

2.5.1 Acaricides and use of drugs

The control of *T. parva* in Tanzania relies on the use of acaricides, commercially available

drugs and vaccination using the ITM (Elisa *et al*., 2015). Treatment with drugs only works during the early stages of the disease. Of all the available drugs buparvaquone is drug of choice for the treatment of *T. parva* (McHardy, Wekesa, Hudson, & Randall, 1985). Recently most pastoralist communities control ECF by limiting ticks using acaricides (Di Giulio *et al*., 2009). However, this method is not of great use due to many drawbacks along with being the development of resistance by ticks, food-safety concerns, disruption of endemic stability and environmental contamination due to the toxic residues. In addition, the use of acaricides involves dipping, and the facilities used for dipping are mostly not operating as they involve financial resources for the maintenance especially among pastoralists who can hardly not afford the cost (Di Giulio *et al*., 2009). The fatal nature of the disease demands for effective measures to provide a sustainable means of controlling the disease which is achieved through vaccination by ITM.

2.5.2 Immunization using the Infection and Treatment Method (ITM)

The cattle normally becomes solidly immune to re infection with similar strains after recovery (Norval *et al.*, 1992). The ITM process involves simultaneous infection of the cattle with three vaccine strains and treatment with oxytetracycline (30%), which results in an attenuate infection but in a long-lasting efficient immune response (Di Giulio *et al*., 2009; McKeever, 2009). The first attempt to vaccinate cattle against *T. parva* infection was done in a year 1911 in South Africa (Theiler, 1912). Whereby they injected the infected cells obtained from spleen and lymph nodes of sick cattle intravenously to the suscpectible cattle, which was not successful and resulted to a lot of death among cattle and consequently vaccination was discontinued (Mbizeni *et al*., 2013).

The original ITM live vaccine technology was developed about 40 years ago (Nene & Morrison, 2016). Protection is mediated by parasite-specific major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTLs), which target the schizont-infected lymphoblast. Because it does not take effect until the schizont parasitosis is established, the CTL response does not prevent infection. The CD8+ T lymphocyte is a response that mediate protection and recently, several parasite antigens recognized by CD8+ T cells have been identified (Pelle *et al*., 2011). In addition, recovered cattle and those immunized by infection and treatment are almost invariably long-term carriers of piroplasm forms. Because piroplasms of *T. parva* undergo only limited replication the carrier state probably arises from persistence of small numbers of schizont-infected lymphoblast's

(McKeever, 2009). Protection is partially stock specific and combinations of stocks have been used to provide broad protection (Oura *et al*., 2007). The most widely used is the 'Muguga cocktail' developed in the 1970s (Radley *et al*., 1975) as a trivalent formulation of *T. parva* strains Muguga, Kiambu 5 and Serengeti-transformed stocks. Over 15 million doses of MC have reportedly been administered in approximately eleven countries of eastern and southern Africa (Perry, 2016) including Uganda, Malawi, Tanzania and, Kenya (Oura *et al*., 2007). Parasite isolates placed in different cross-immunity groups usually exhibit 20–30% cross-protection between them (Morzaria, Spooner, Bishop, & Mwaura, 1997). Sequential immunization by ITM with single isolates in different cross-immunity groups provides additive immunity (Taracha, Goddeeris, Morzaria, & Morrison, 1995). This suggests that some protective antigens are shared between different parasite isolates, and the breadth of the immune response can be expanded in response to new infections (Nene *et al*., 2016). Novel genotypes can be found in cattle vaccinated with the Muguga cocktail (Hemmink *et al*., 2016; Oura *et al*., 2004). This may help to broaden the spectrum of immunity to strains of the parasite not present in the cocktail.

The results of two current studies of the Muguga cocktail vaccine, one involving genomic sequencing of the three component parasites and the other based on high-throughput sequencing of PCR amplicons of six genes encoding *T. parva* antigens (including Tp1 and Tp2), have indicated that the vaccine contains only a small component of the genetic and antigenic diversity detected in field populations of *T. parva* (Nene *et al*., 2016). Each of the three parasite isolates in the Muguga cocktail showed a very limited diversity, and two of them (Muguga and Serengeti) exhibited a striking high level of sequence similarity, but differed significantly from the Kiambu isolate (Nene *et al*., 2016).

Furthermore, amplicon sequencing and satellite DNA typing indicated that the vaccine components contained minor genotypic components present at <5% within the vaccine parasites. If these minor components contribute to the broad protective capacity of the vaccine, then the possibility that these components might not be present in all vaccine batches or indeed all vaccine doses is of concern regarding standardization of vaccine content (Nene *et al*., 2016).

The greatest impact of the MC appears to have been in Tanzania, in part due to the commitment of the group championing its use in and around Arusha, and in part due to the unique demand in the pastoralist communities in northern Tanzania and southern Kenya

(Perry, 2016). As the Muguga Cocktail is a live vaccine there has been concerns about the use of it due to the risk of introducing exotic stocks of *T. parva* from the vaccine parasite strains (or specific alleles) into the field which maybe outside the immunological range of indigenous parasites (Oura *et al*., 2007) this may result in genetic recombinations with local parasite populations or introduction of the disease in previously free areas (Martins *et al*., 2010).

Moreover vaccination using the Muguga cocktail requires production of three large batches of *T. parva* sporozoites by feeding ticks on cattle infected with each parasite isolate, and each batch needs to be carefully titrated in cattle to determine a dose that will reproducibly infect and immunize all animals but will not break through the tetracycline treatment (Nene *et al*., 2016). This complex protocol coupled with the requirement for a liquid nitrogen cold chain to distribute the vaccine presents drawbacks for quality control and marketing. Nevertheless, recent initiatives have led to increased field uptake. This has included the establishment of a center for vaccine production and systems to facilitate distribution of the vaccine (Nene *et al*., 2016). Therefore, development of a subunit vaccine that is easier to produce and with minimal risks is important. Previous experiments with other versions of the ITM vaccine have shown that they do not always provide complete protection under field conditions (Cunningham *et al*., 1974). A number of cross-immunity studies have been conducted using different cattle-derived *T. parva* stabilates, including locally derived parasites, which have shown mixed results in development of protective immunity (Latif, Hove, Kanhai, Masaka, & Pegram, 2001).

2.6 Carrier state and persistence of Muguga Cocktail vaccine

The carrier state of *T. parva* is the ability of an infected and recovered host to carry the parasites without getting sick but it can infect ticks which are then able to transmit the parasite to susceptible animals (Norval *et al*., 1992). The cattle which recover from the disease can remain infected with no clinical signs and serve as reservoirs for ticks (Olds *et al*., 2018). The phenomena of carrier state of *T. parva* among cattle contributes to continuous infection of cattle which plays a mojor role in maintenance of cattle immunity (Young, Leitch, Newson, & Cunningham, 1986). Carrier state is also said to result into endemic stability, in areas where natural transmission occurs and is described as "a climax relationship between host, agent, vector and environment in which all coexist with the virtual absence of clinical disease" Woolhouse *et al*. (2015). Calves are most susceptible to

acute infections under conditions of endemic stability and the developed carrier state may last for a number of years. Lately the concept of endemic stability for *T. parva* has been defined to include the concept that protection may be correlated with closely related virulent *Theileria* species which circulates naturally among the cattle population (Olds *et al*., 2018). It is therefore very important to understand the contribution to cattle immunity made by persistent *T. parva* infections both naturally acquired and artificially induced through vaccination with live sporozoites given the broade concept of endemic stability (Olds *et al*., 2018).

Undisrupted endemic stability helps to reduce the occurrence of acute disease and mortality (Norval *et al*., 1992). Movement of naive cattle from non endemic to endemic zones together with the breakdown of tick control may reduce transmission pressures, as this may limit the opportunity for early infection of calves and subsequent development of a broad immune response. Moreover, herd improvement programs that introduce more productive European cattle breeds which are highly susceptible to infection, or by the introduction of virulent *T. parva* strains directly from African buffalo reservoirs at the interface between domestic cattle and wildlife can also disrupt the endemic stability (Olds *et al*., 2018). Epidemiology of theileriosis is determined by the ability of the *T. parva* carrier animal to infect ticks during feeding. There are two main ways by which a mammalian host can become a *T. parva* carrier: spontaneous recovery from an infection without treatment or a recovery after treatment. The ITM method against *T. parva* infections has been very effective in conferring the cattle immunity but these cattle remain carriers (Mbizeni *et al*., 2013).

It is possible for the animal that is infected with *Theileria* parasite to act as a carrier only initially, i.e. develop schizont parasitosis and piroplasm parasitaemia which are later cleared by the immune responses leaving the host immune but with no parasites producing the state of sterile immunity. Thus an animal can become an intermittently or sporadic carrier or become a persistent carrier (Norval *et al*., 1992).

An infected mammalian host, either buffalo or cattle, should be capable of infecting ticks which then transmit the parasites to a new host this is a crucial stage in *Theileria parva* maintenance in cattle populations which results to a continuous circulation of the parasite between the mammalian host and the vector ticks (Norval *et al*., 1992; Mbizeni *et al*., 2013). The above case can only be achieved if there is constant contact between infected

mammalian hosts and susceptible cattle (Mbizeni *et al*., 2013). Physical separation has been effective before as it limits contact between known infected cattle or buffalo and susceptible cattle which may also contribute to inability for buffalo-derived *T. parva* to establish carrier state in cattle populations which is not the case in northern Tanzania due to animals co grazing and pasture sharing at wildlife interface areas which may expose animals to buffalo derived strains. Moreover, all stages of *R. appendiculatus* occur throughout the year in tropical East Africa which makes continuous host-vector *T. parva* circulation possible (Mbizeni *et al*., 2013).

The study done by Oura *et al*. (2007) revealed the persistence of one of the three strains known as kiambu 5 to be detected in the majority (70%) of vaccinated calves for up to 4 years, but there was no evidence for the presence of the Muguga or Serengeti component of the vaccine which suggest that some components in Muguga stock does not cause a long term carrier state (Skilton *et al*., 2002). The use of a live vaccine means that vaccinated animals become "carriers" of the *T. parva* strains being used in the vaccine, and eventually they then provide a source of these strains to ticks in the field as a result they have the potential to infect co-grazing non-vaccinated animals with these strains, this raises many concerns on the MC vaccine (Hayashida *et al*., 2012).

The study done by Oura *et al*. (2007) suggested that the Kiambu 5 stock can be detected in many but not all vaccinated cattle for up to 4 years and can be transmitted to unvaccinated cattle which share grazing, and that some of the vaccinated animals become infected with local genotypes without developing into a disease. This possibility was examined by collecting of blood samples from 13 unvaccinated adult cattle on the farm (over 2 years after the previous sampling) including 3 of the animals sampled in 2002. These cattle had shared grazing for over 4 years with 43 cattle, vaccinated between 2000 and 2003. Blood samples were genotyped, using the Muguga/Serengeti and Kiambu 5-derived primers that amplify regions of the PIM gene. Four of the unvaccinated cattle (numbers 3, 8, 10 and 12) amplified PCR products of the same size and pattern as the Kiambu 5-specific PCR products from the vaccine stabilates.

2.7 Genotypic diversity of *T. parva*

Like other parasites protozoan parasites are thought to have evolved in genetic diversity to survive the immunologically unfavorable environments of their hosts (Nene *et al*., 2016). Genetic diversity allows the parasites to escape the immune responses of their hosts

due to antigenic variation in parasites (McKeever, 2007). Recombination which takes place in ticks during sexual reproduction is very crucial in the genetic diversity of *Theileria* species (Henson *et al*., 2012; Katzer, Ngugi, Schnier, Walker, & McKeever, 2007) although other mechanisms like genetic drift and mutations also accounts for this (Skilton *et al*., 1998), Bioinformatics analyses have revealed this to be a possible mechanism generating genetic diversity in genes such as the polymorphic immunodominant molecule (PIM) of *T. parva* (Geysen *et al*., 2004; Sivakumar, Hayashida, Sugimoto, & Yokoyama, 2014). Besides genetic recombination and mutations within the epitopes of CD8+ cytotoxic T lymphocyte (CTL) antigens were found to facilitate immune evasion in *T. parva* (Connelley, MacHugh, Pelle, Weir, & Morrison, 2011). Whilst the evolutionary acquisition of genetic diversity favors the long-term survival of the parasites, it usually makes it difficult to adopt to efficient control measures against the diseases caused by them. Therefore, this calls for a deeper knowledge of genetic diversity in *T. parva* for gaining better understanding of these harmful parasites (Sivakumar *et al*., 2014).

Studies influenced by the distribution, density, and type of markers across the genome by using a number of loci and variable number of tandem repeat (VNTR) markers (mini- and micro- satellites) derived from the *T. parva* genome sequence data have been used as markers to determine parasite diversity (Oura *et al*., 2003, 2004, 2005, 2011; Patel *et al*., 2011) the results indicated that there was no direct relationship between geographical origin and level of genetic similarity between parasite isolates, such that different parasite isolates from the same farm demonstrated distinct genotypes. Older cattle revealed larger number of different parasite genotypes than younger ones, which tended to have a predominating genotype (Oura, Asiimwe, Weir, Lubega, & Tait, 2005). Some geographical sites revealed a sub structure in parasite populations, others did not, and some exhibited an epidemic structure characteristic of recent predominating infections (Oura *et al*., 2005). Thus, it has been concluded that cattle movement, their co grazing with buffalo, and parasite transmission rates play a major role in determining *T. parva* parasite population structures (Oura *et al*., 2003).

Analysis with 12 micro and minisatellites markers revealed 84 multilocus genotypes (MLGs) in blood samples from three geographical localities in Uganda (Oura *et al*., 2005), and a total of 183 alleles were observed at 30 micro and minisatellites loci from 20 Kenyan tissue culture isolates (Odongo *et al*., 2006). Some animals can be infected with a couple of

T. parva genotypes (Oura *et al*., 2007). Even so most recent analysis of 14 micro- and minisatellites markers revealed a low level of genetic diversity in *T. parva* from cattle populations in Tanzania (Elisa *et al*., 2015; Rukambile *et al*., 2016). Other findings of the study done in south Sudan using a panel of 11 mini- and three micro-satellite markers revealed high *T. parva* diversity in some parts (Salih *et al*., 2018). These observations are similar among *T. parva* populations in countries where ECF is endemic.

2.7.1 Influence of wildlife interface areas in *T. parva* diversity

Ticks plays a major role in transmission of *T. parva* parasites among the vaccinated and unvaccinated animals which co graze and with close proximity to wildlife interface where there is constant exposure with the buffalo which is the main host of the parasites, in spite the fact that wildlife harbors a great diversity of parasites in inactive forms they still have a potential to cause drastic effects on their hosts both directly and indirectly (Wamuyu *et al*., 2015). Once the cattle are infected with buffalo-derived strains it develops a Corridor disease (CD), which is results to low levels of parasitized leukocytes in peripheral lymph nodes unlike the high parasitosis seen in ECF. Parasites derived from buffalo are more divergent compared to the ones from cattle this is evidenced by the monoclonal antibody profiles results and comparison of the sequences of known *T. parva* antigens (Pelle *et al*., 2011). Moreover, previous studies in many parts of Africa, including Zimbabwe (Bishop *et al*., 1994), Uganda (Oura, Tait, Asiimwe, Lubega, & Weir, 2011) and South Africa (Sibeko *et al*., 2011), reported the same findings. Several genes have been investigated in search of discriminatory sequence differences between *T. parva* isolates. Among these are sporozoite antigen genes, p104, p67 and PIM (Bazarusanga *et al*., 2007; Iams *et al*., 1990; Nene, Musoke, Gobright, & Morzaria, 1996). The heterogeneity seen in *T. parva* is unclear whether it exert any advantage during the stages of the parasite lifecycle. Notwithstanding that, there is evidence that this diversity has an important influence on immunity (Sitt *et al*., 2015). Immunity resulting from Muguga cocktail is known to be strain-specific hence cattle immunized against one strain of the parasite are not necessarily protected against challenge with heterologous parasite strains in the field (Radley *et al*., 1975).

The Muguga cocktail vaccine has been used successfully in some regions despite the evidence that it does not protect all animals against infections from buffalo derived strains (Radley *et al*., 1979). Two studies were conducted among vaccinated cattle grazing into areas where buffalo resides showed no or low levels of protection against disease (Bishop *et*

al., 2015; Sitt *et al*., 2015). The findings suggests that *T. parva* parasite populations in buffalo harbor a greater antigenic diversity than those in cattle (Hemmink *et al*., 2016). To support these findings a study of the sequences of two genes (Tp1 and Tp2) which encode proteins recognized by CD8+T cells from immune cattle, in infected cell lines isolated primarily from eastern Africa (Pelle *et al*., 2011). Over 30 allelic variants of each antigen were identified, majority of the variants were found in isolates obtained from buffalo and from cattle that had co grazed with the buffalo, whereas only a small amount of the variants was detected in the isolates obtained from cattle grazed without the buffalo (Pelle *et al*., 2011).

Recent studies done by Elisa *et al*. (2015) to investigate the genetic diversity of *T. parva* isolates existing in cattle and African buffalo in the Serengeti and Ngorongoro in Tanzania and whether *T. parva* genotypes are shared between buffalo and cattle that are grazing in same pasture or in close proximity, showed a higher incidence of *T. parva* and buffalo derived strains to have been transmitted to Ngorongoro cattle which are co-grazed in close contact with buffalo as compared to Serengeti cattle.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study areas

The study was carried out in Maasai steppe of northern part and Tanga region in the eastern part of Tanzania (Fig. 2). The Maasai steppe is made of Simanjiro plains, Tarangire National Park and Lake Manyara National Park. It stretches over right districts of Arusha and Manyara regions of Tanzania. It lies between 3°52' and 4°24' south and 36°05 and 36°39 east. This area has two rainfall seasons of spatial and temporal variation. It is comprised of dry season starting from October to December and wet season from February to May. The average temperature in the area is between 18°C and 30°C. The Maasai steppe is made up of natural ecosystem consisting of a variety of animal species, vegetation, conducive temperature variations and rainfall, all of which support a natural habitat for living organisms including vectors and various parasites. Such an ecosystem regulates the density of organisms found in the area. The area is semi-arid with the national parks providing a home for wildlife. The main source of livelihood in Maasai steppe is Livestock and crop production with cattle production being the major activity. This area also consists of a wildlife corridor which is bordered by Monduli district and Manyara ranch located in the north and Tarangire National park on the South-western side. Due to its proximity to wildlife the area has high interaction of domestic and wildlife which increases circulation of *T. parva* making East Coast Fever endemic in the area.

Tanga Region is one of the 31 administrative regions of Tanzania. It is bordered by Kenya and Kilimanjaro Region to the north; Manyara Region to the west; and Morogoro and Pwani regions to the south. Its eastern border is formed by the Indian Ocean. It is situated at the North-East corner of Tanzania between 4◦ and 6◦ degrees below the Equator and 37◦ -39◦ 10' degrees East of the Greenwich Meridian. Tanga's climate is classified as tropical, with the average temperature of 26 °C. About 1290 mm of precipitation falls annually, with the greatest amount occurs in May at an average of 294 mm which supports the parasites survival including ticks Most of the land is used for crop production, livestock keeping, forestry, mining, and residency. Livestock keeping is the second important sector of the economy in the region. Over 94% of livestock kept in Tanga constitute traditional/local breeds which have very low potential in terms of production and productivity. According to the census of Agriculture survey in 2007-2008 the region has a

total of 732 130 cattle in 74 670 households. Most of the households keeps indigenous cattle (688 114), which accounts for 94% of the total cattle population. With beef cattle accounting for only 0.3% of the cattle while the remaining households raises a total of 41 639 dairy cattle, which comprises 6% of the cattle population. Unlike the Maasai steppe the dairy farms in Tanga region are located far from the wildlife interface areas hence there is no interaction of the domestic animals with the wildlife. Furthermore, in Tanga region ITM practice has not been practiced for a longer time as compared to the Maasai steppes.

Figure 2: The map of Tanzania showing key sampling sites

3.2 Study design

A cross sectional study design was used to obtain the required results in which sampling was conducted in five villages in the Maasai steppe of northern Tanzania and two farms in Tanga region in March 2018 which was the wet season characterized by the presence of many ticks. Bomas (traditional Maasai homesteads, usually consisting of a number of huts surrounding an enclosure for cattle) were the sampling unit.

3.3 Sample size determination

Sample size was calculated using the following formula

$$N = \frac{(Z\alpha)^2(pq)}{d^2}$$

Where N is the sample size; Zα =1.96 at 95% confidence level; standard error of the mean, p= 37.1%, the previously reported *T. parva* prevalence in the area (Kazungu *et al*., 2015); q=1-p, d=0.05 at 5% absolute error (design effect).
The calculated sample size was 358

3.4 Sampling

A multistage sampling technique was used from the district level to the bomas which were the sampling units. Five villages in Maasai steppe and two farms in Tanga were then selected purposively based on their cattle population size, location with reference to Tarangire and Manyara National park and whether ECF vaccination is being practiced. In these locations all of the sub-villages were listed down and 50% of them were selected randomly. In each of the sub- village the numbers of the bomas were listed down and 3 bomas were selected systematically in each sub village. Cattle were then selected conveniently in the selected bomas. Vaccinated cattle were easily identified by ear-tag numbers, which indicated the year of vaccination what was also confirmed by farmers. Upon receipt of consent from local and village authorities as well as individual farmers, cattle were randomly selected from seven locations (Loiborsoit, Emboret, Esilalei, Mswakini, Manyara ranch, Tanga Leila farm and Tanga Mruazi Farm).

Data on their vaccination status and duration since vaccination were retrospectively collected. Mruazi and Leila farms in Tanga were selected purposively due to their exclusiveness from the wildlife and the ITM practices. Mruazi farm had never practiced ITM whereas, Leila farm stopped vaccination in 2015. These farms become good candidates for comparison of impact of ITM from the Maasai steppe areas where it has been practiced for over 15 years and close to wildlife. A sample size of 336 cattle was enrolled in order to determine the carrier state persistence and diversity of *T. Parva.* Twenty two of the cattle dropped out during sample collection.

3.4.1 Blood samples collection

Blood samples were collected from the jugular vein using 10 ml EDTA vacutainer tubes (Becton Dickson Vacutainer Systems, England). Blood samples were collected from villages and farms as detailed in Table 1. The blood samples were labeled and stored in a cool box with ice packs while in the field and later put into a refrigerator until when they were transported to NM-AIST laboratory. Blood samples for DNA extraction were kept frozen at -20°C before shipped to Sokoine University of Agriculture for analysis.

Table 1: Total number of blood samples collected per location

Study site	Number of animals sampled
Loiborsoit	35
Emboreet	41
Esilalei	69
Manyara Ranch	72
Mswakini	63
Leila Farm	36
Mruazi Farm	20
Total	336

3.5 Laboratory analysis

Laboratory analyses were conducted in the Genome Science Center at the Faculty of Veterinary Medicine at Sokoine Univesity of Agricuture, Morogoro.

3.5.1 DNA extraction

DNA was extracted from cattle blood using the, Quick-g DNA™ Blood miniprep (D 3073, Zymo Research, USA). Prior extraction, blood samples were left to thaw at room temperature for three hours. 100 µl of blood was put in a 1.5 ml micro-centrifuge tube, followed by 400 µl of genomic lysis buffer (containing 0.5% beta-mercaptoethanol). The contents were mixed thoroughly at 20 Hertz for five seconds on a vortex (VELP scientifica) and incubated at room temperature for 10 minutes. The mixture was then transferred to a Zymo-spin column in a collection tube and centrifuged at 10 000 rpm for one minute (Eppendorf 541R, USA). The collection tube was then discarded with the

flow- through solution. The Zymo-spin column was then transferred to a new collection tube and 200 µl of DNA Wash Buffer was added to the spin column and centrifuged at 10 000 rpm for one minute. The spin column was then transferred to a clean and sterile micro-centrifuge tube where DNA was eluted by adding 50 µl of DNA Elution Buffer and incubated for five minutes at room temperature. Then the spin column was discarded and DNA collected in a micro-centrifuge tube. The eluted DNA was then run on gel to see whether it was successfully extracted.

3.5.2 Nested PCR for screening *T. parva* positive samples using p104 gene

All samples were screened for *T. parva* using a nested polymerase chain reaction (PCR) assay targeting the 104 kDa antigen (p104) gene (Skilton *et al.,* 2002) The assay was run in two PCR rounds; the primary and secondary PCR; using outer primers for primary PCR (For1 '5-ATTTAA GGA ACC TGA CGT GAC TGC-3') and (Rev1 '5-TAA GAT GCC GACTAT TAA TGA CACC-3'), and inner primers for secondary (nested) PCR (For2 '5-GGC CAA GGT CTC CTT CAG AAT ACG-3') and (Rev2 '5-TGGGTG TGT TTC CTC GTC ATC TGC-3'). Primers were designed based on p104 antigenic gene (Gene bank M29954).

The primary PCR composed of 6.25 µl Master mix, 3.25 µl nuclease free water, 0.25 µl of each of the primers (For and Rev) and 2.5 µl of gDNA to have a final volume of 12.5 µl. The reaction was briefly vortexed and then centrifuged for 1 minute at 14 000 rpm to bring all the droplets down. The nuclease free water was used as negative control and the DNA sample known to be *T. parva* positive from BecA-ILRI Hub sample repository was used as positive control. The amplification conditions for primary PCR were 95℃ for 1 minute followed by 30 cycles of 95℃ for 1 minute, 60℃ for 1 minute, 72℃ for 1 minute with additional 10 minutes at 72℃ as final extension. The components of the secondary PCR remained the same as for the primary PCR except the template which was primary PCR products diluted at 1:10. The amplification conditions were the same except the annealing temperature and the number of cycles was reduced to 55℃ and 30 cycles, respectively. All amplifications were done using a programmable thermal cycler (MJ Research, Watertown, MA, USA). The secondary round PCR products were analyzed by electrophoresis and run at 100 V for 40 minutes in 1.5% agarose gel.

3.5.3 PCR amplification and analysis of mini and microsatellite loci

The nested PCR deploying the outer and inner primers designed by Oura *et al.* (2003) and Salih *et al.* (2018) respectively (Table 2) was performed to amplify each of 1 mini and 2 microsatellites used in the study for each sample that was positive by p104 nested PCR amplification. The primary PCR amplification was done in 10 µl comprising of 2 µl of 20 ng/µl genomic DNA, 5 µl of Master mix, 0.4 µl of each outer primers at 10 pmole and 2.2 µl of nuclease free water. The nuclease free water was used as negative control and the DNA sample known to be *T. parva* positive from BecA-ILRI Hub sample repository was used as positive control. The cycling conditions for the primary PCR were as follows; Initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, extension at 72°C for 1 minute plus a final extension at 72°C for 10 minutes. For the secondary PCR, all other reagents remained the same except that 0.5 µl of the primary PCR was used as the template and the volume of water was increased accordingly to give a total of 10µl reaction volume. The cycling conditions for the secondary PCR were as follows, Initial denaturation at 95°C for 5 minutes followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 1 minute, extension at 72°C for 1 minute plus a final extension at 72°C for 20 minutes. Five microliter of the amplicons were analyzed on a 1.5% agarose gel to check for amplification success.

Table 2: Panel of mini and microsatellite markers used to genotype *T. parva* samples used in this study

Markers		Outer nested primers sequence	Inner nested primers sequence
MS 7	**For**	CTCCTCAGCATCCTGCTGCTCATTG	GTTCAGTCCTATGGCAATTCAG
	Rev	GCGCATGACTGCTTTTACATTAACCC	CAAACCTCTTCAAATTCACTCTAGG
ms 5	**For**	AACACAGTAACTAACCCAGGCC	AATCTTCCAATCCCAACCACATAC
	Rev	AACTCCAGCGGAATCCCGAAATA	CCCGAAATAAAACCAAATTCCACC
ms 2	**For**	AAGTTAGTATCACCACCAGGCTGG	GCCCAATGTACCGAGAATCCTCAC
	Rev	GGCTCATCTACCACTCCAACTCC	ATTCTCCGCATTCTCCACCACCTC

3.5.4 Agarose gel electrophoresis

PCR products were loaded in 1.5% agarose gels prepared in TAE buffer. The products were separated at 80 volts for 40 minutes before visualization and documentation on Gel Doc TM (Bio Rad, USA).

3.5.5 Data analysis

Data were entered and cleaned using Microsoft excel 2016. Vision capt software version 15.0 was used for scoring of allele sizes from gel pictures. GenALEX software version 5 was used to calculate genetic diversity parameters for the entire dataset. This included determining the number of alleles per locus (A), and expected heterozygosity (He). These parameters were used to assess the level of polymorphism at each locus and determine diversity overall and within the sub-populations. Expected heterozygosity was calculated using the formula for 'unbiased heterozygosity' also termed haploid genetic diversity, $He = [n/ (n-1)] [1-\sum p2]$ where n is the number of isolates and p the frequency of each different allele at a locus. Analysis of molecular variance was used to study the *T. parva* population in the study locations by giving the percentage variations between and within the parasite populations. Principal component analysis (PCA) was used to investigate the genetic relationships between the *T. parva* isolates from different geographical locations used in the study. Descriptive statistics were computed at 95% Confidence Interval (CI). Chi-square test was used to determine association between outcome variables (*T. parva* carrier state) and categorical variable such as vaccination status and grazing close to the wildlife interface areas. Statistical significance was determined at $p< 0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Screening for *T. parva* carrier state in cattle by p104 nested PCR

Three hundred and thirty six (336) cattle were screened for *T. parva* carrier state using p104 nested PCR (np104). A 277bp PCR product was observed for 116 (34.5%) cattle, which were henceforth considered as *T. parva* carriers. The proportion of carriers was significantly higher among vaccinated cattle (43%; 103/239) compared to the unvaccinated cattle (13.4%; 13/97) (p<0.000). Likewise, a higher frequency of *T. parva* positivity was documented among cattle grazing in closer proximity to wildlife 38% (107/280) than cohorts grazing farther from wildlife 16% (9/56) (P<0.001). The proportions of cattle, categorized based on p104 PCR results are shown in Table 3.

Table 3: Proportions of cattle categories based on *T. parva* carrier status

Category	Sub-category	*T. parva* infection status		Total	P value
		Positive, n (%)	Negative, n (%)		
Vaccination Status	Vaccinated	103 (43.1)	136 (56.9)	239	P<0.000
	Unvaccinated	13 (13.4)	84 (86.6)	97	
Proximity Wildlife	Close	107 (38.2)	173 (61.8)	280	P<0.001
	Far	9 (16.1)	47 (83.9)	56	
Overall		116 (34.5)	220 (65.5)	336	

Vaccinated cattle are more likely to be *T. parva* carriers 5 times than the unvaccinated cattle (Odds Ratio= 4.89) and the cattle grazing near to the wildlife interface are 3 times more likely to be *T. parva* carriers than the ones grazing far from the interface (Odds Ratio= 3.18).

Table 4 shows the prevalence of *T. parva* between cattle groups split by their vaccination status. Although sampling of cattle was random, but the proportion of vaccinated and unvaccinated cattle varied between the populations. Thus, the sampling emerged with approximately equal proportions of vaccinated and unvaccinated cattle in two

populations, Tanga Leila farm and Loiborsoit, but in some populations, Manyara ranch and Mswakini all cattle were ECF- vaccinated, while none of the cattle were ECF-vaccinated in Mruazi group. Accordingly, the overall prevalence of carrier state varied between the cattle groups and was highest in Manyara ranch (82%) and lowest in Mruazi farm (0%). While majority of the carriers were vaccinated animals, 13 of the carriers were not ECF-vaccinated and were sampled from different groups (Leila farm, Loiborsoit and Esilalei groups each with 4, 2 and 7 cattle.

Table 4: Prevalence of *T. parva* among the six groups used in this study

Locations	Vaccination Status	N	*T.parva* positive	Prevalence, %	Overall prevalence, %
Leila farm	Vaccinated	17	5	29	25
	Unvaccinated	19	4	21	
Loiborsoit	Vaccinated	16	3	18.75	14.3
	Unvaccinated	19	2	10.5	
Emboreet	Vaccinated	35	9	25.7	13
	Unvaccinated	6	0	0	
Manyara Ranch	Vaccinated	72	59	82	82
	Unvaccinated	0	0	0	
Esilalei	Vaccinated	29	14	48.3	30.4
	Unvaccinated	40	7	17.5	
Mswakini	Vaccinated	63	14	22.2	22.2
	Unvaccinated	0	0	0	
Mruazi farm	Vaccinated	0	0	0	0
	Unvaccinated	20	0	0	
Overall	Vaccinated	239	103	43	28.2
	Unvaccinated	97	13	13.4	

4.1.2 Persistence of carrier state following ECF vaccination

The p104 PCR successfully detected *T. parva* carrier state in 103 (43%) out of 239 cattle, that had been immunized at variable time points between 4 months and 13 years earlier before blood sampling was done for present study. Although carrier state was detected in

cattle early after vaccination, but highest abundance of carrier cattle was documented between 8 and 12 months post vaccination. The carrier state persisted in vaccinated cattle and was detectable by the nested PCR as long as 11 years post ECF vaccination (Fig. 3).

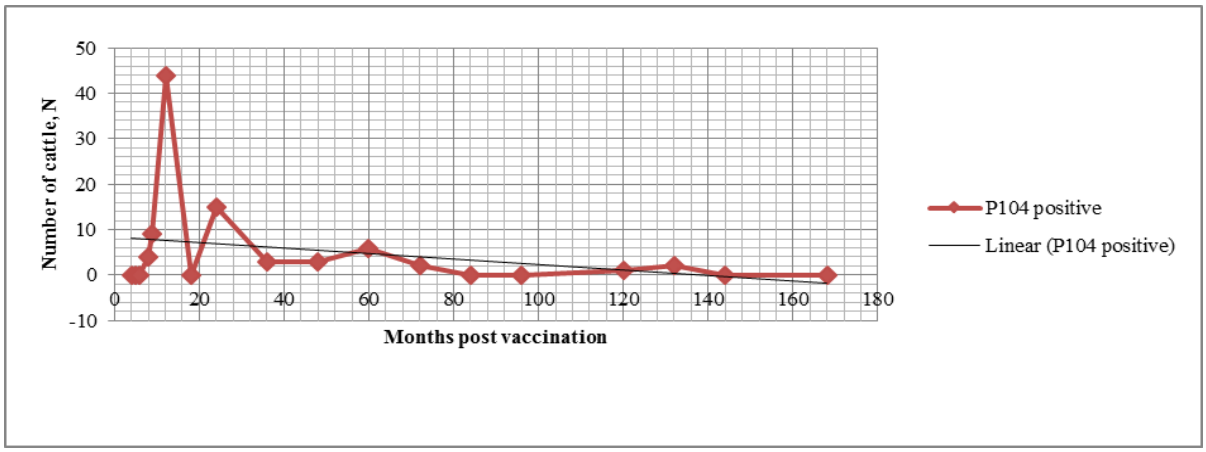

Figure 3: Scatter plot showing the persistence of carrier state over time since ECF vaccination

4.1.3 Detection, prevalence and distribution of mini- and micro-satellite markers in cattle

Three VNTR (Variable nucleotide tandem repeats) Minisatellite 7 (MS7), microsatellite 5 (ms5) and microsatellite 2 (ms2) markers were selected to monitor the prevalence and persistence of the ECF (Muguga) vaccine in cattle. Only the 116 cattle, previously confirmed by PCR to be *T. parva* carriers were subjected to this analysis. Cattle blood samples positive for these markers revealed different band sizes after running them on agarose gels for documentation as shown in (Fig. 4).

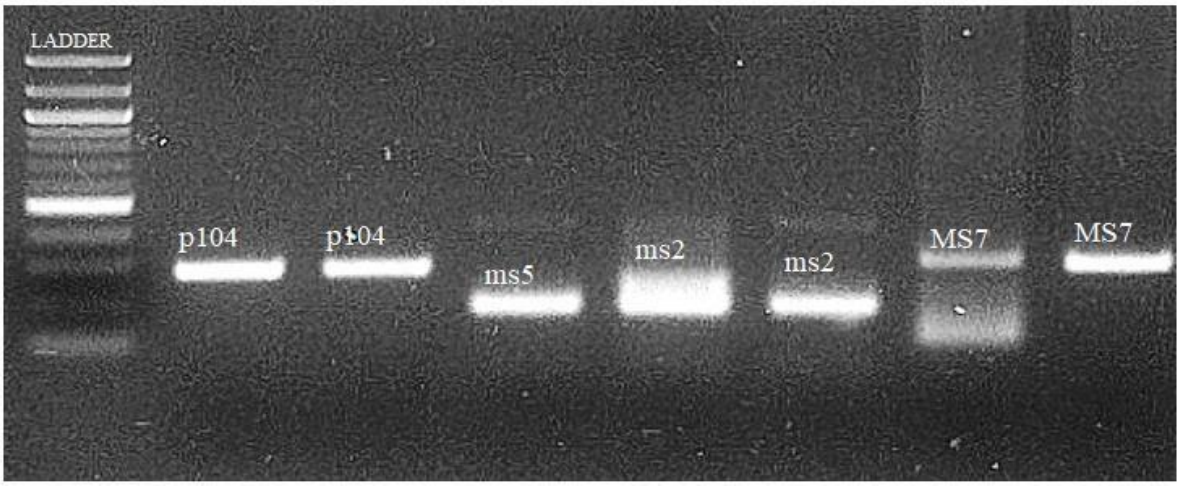

Figure 4: Agarose gels (1.5%) showing some of PCR products generated using three VNTR markers and a p104 gene to amplify *T. parva*

All three markers were polymorphic in the set of samples as evidenced by the observation of more than one allele at either locus. Distribution and prevalence of the three markers in the study population is summarized in (Fig. 5) in the subpopulation of 116 *T. parva* carriers ms 5 marker was predominant (42%) followed by MS 7 (41%) and ms 2 (15%) respectively, ms 2 was not detected in the unvaccinated group.

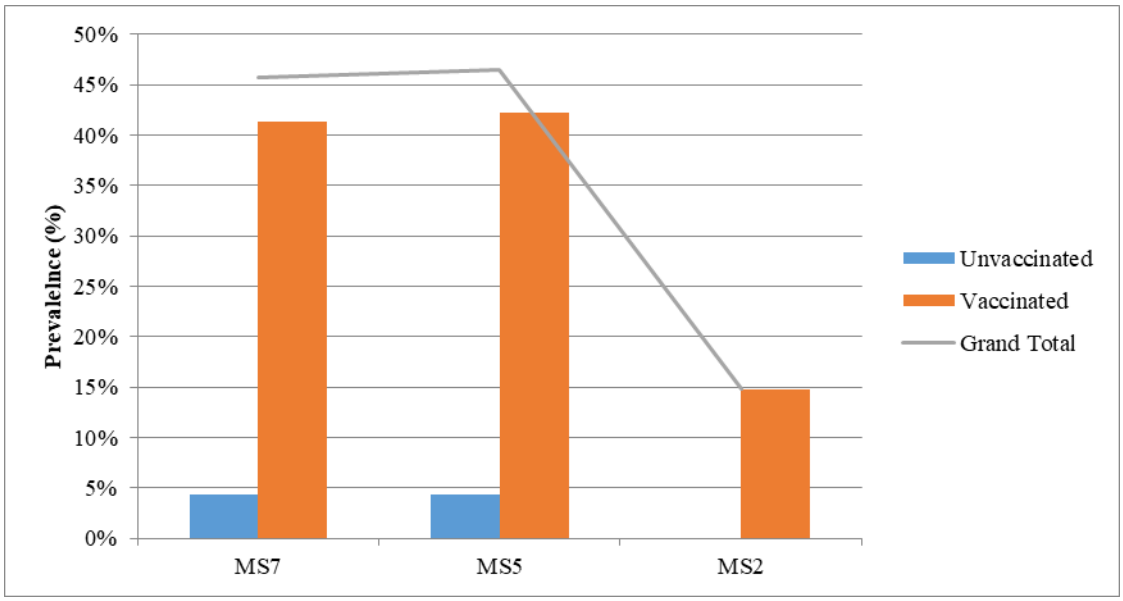

Figure 5: The distribution of the three markers in vaccinated and unvaccinated cattle

4.1.4 Analysis of genetic diversity of *T. parva* using 3 VNTR markers

Genetic diversity among the previously identified *T. parva* carrier cattle was characterized using 3 VNTR markers shown in Table 5. Of the three markers ms 5 had the highest number of alleles (n=10) whereas MS 7 had the least number of alleles (6). Generally, allele sizes across the three markers ranged from 100 bp to 450 bp and percentage polymorphism ranged from 75 to 100.

Table 5: The number of alleles and percentage polymorphism of vaccine markers

Variables	MS 7	ms 5	ms 2
Total analyzed, n	53	56	16
Number of alleles	6	10	8
Size range, bp	150-450	100-220	100-260
% polymorphic loci	100	83.3±16.7	75±25

The distribution of alleles of the three markers (MS 7, ms 5 and ms 2) were observed in all cattle groups with variable magnitudes. Microsatellite 5 was detected across all six cattle groups while ms 2 detected in four groups. Alleles with highest frequency were those with 200bp (ms 5) in Loibosoit, 300bp (MS 7) in Leila, Manyara ranch and Esilalei while 450bp (MS 7) was detected in Emboret (Fig. 6a, 6b and 6c).

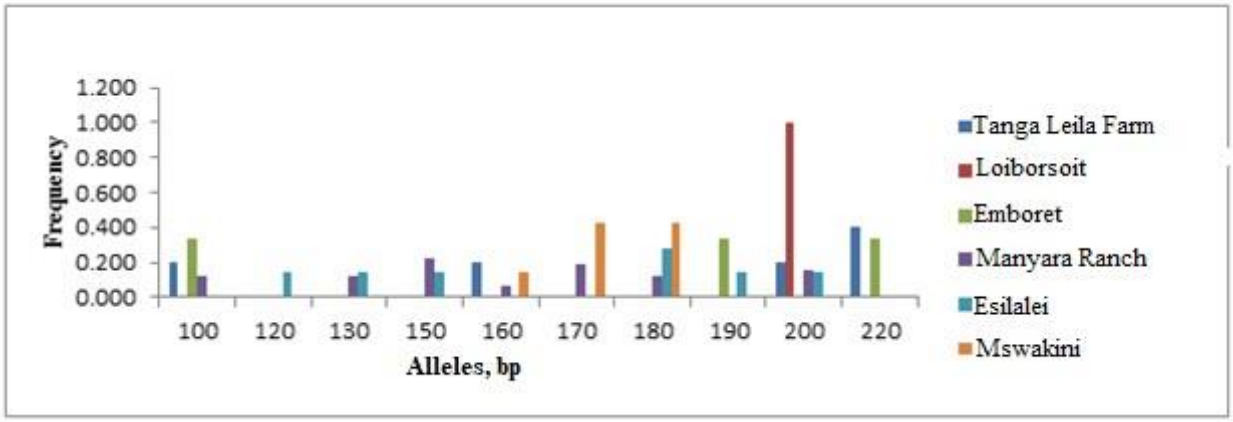

Figure 6a: Distribution of ms 5 alleles across the study locations

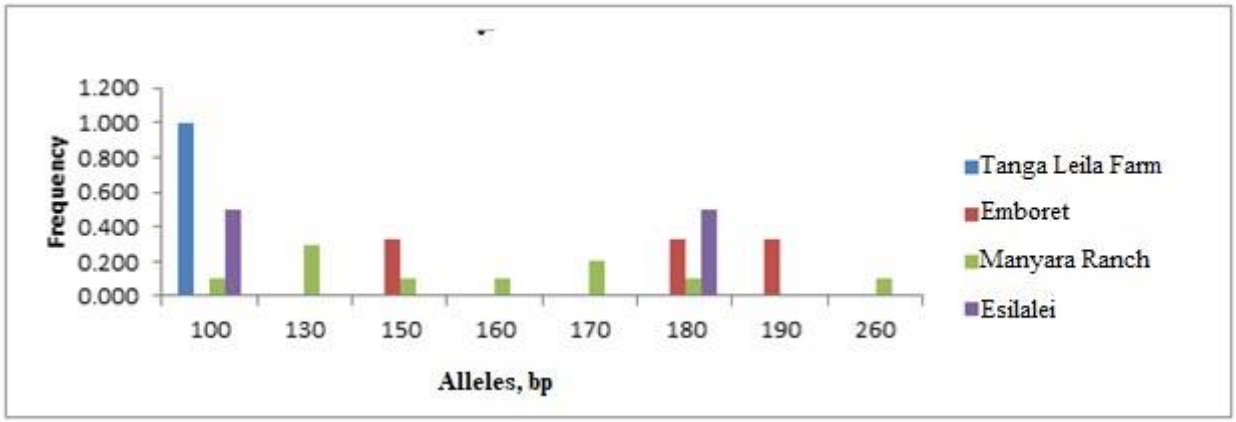

Figure 6b: Distribution of ms 2 alleles across the study locations

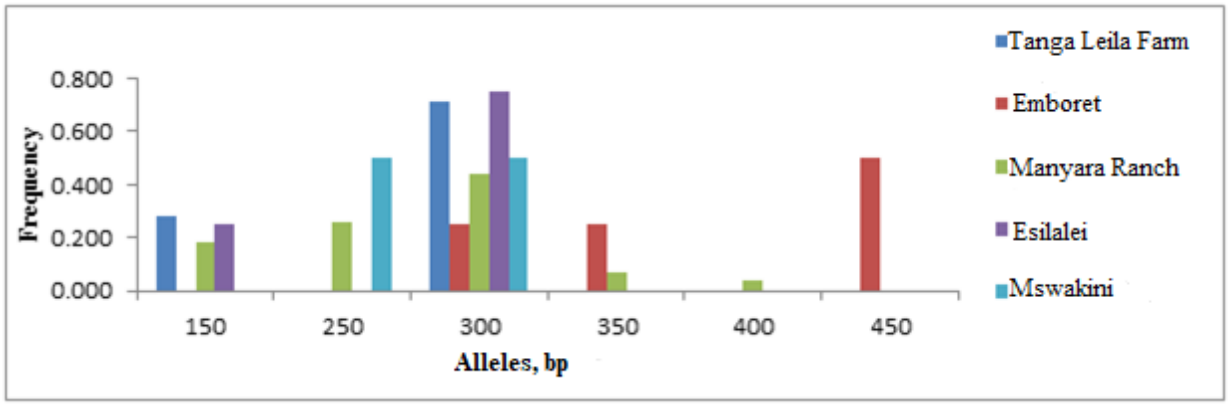

Figure 6c: Distribution of MS 7 allele across the study locations

Analysis of the haploid allelic frequencies further revealed presence of private alleles in all markers (Table 6) with four ms 2 private alleles observed in Esilalei.

Table 6: Private alleles observed in different cattle groups

Markers	Private alleles (n)	Size range (bp)	Groups
ms 5	1	120	Esilalei
ms 2	4	130, 160, 170, 260	Manyara ranch
MS 7	2	400, 450	Manyara ranch, Emboret

Although the total number alleles detected in each marker ranged between six and ten but when the cattle were split based on vaccination status, only four alleles were detected in unvaccinated cattle two each for ms 5 (200bp and 220bp) and MS 7 (150bp and 300bp) markers and none for ms 2. Out of 116 *T parva* cattle carriers, 12 had all the three markers with 300bp MS 7 allele depicted high frequency (9/12) Fig. 7. Moreover, all the 12 cattle were from the vaccinated category and were sampled from Emboret, Esilalei and Manyara ranch, which are all close to wildlife.

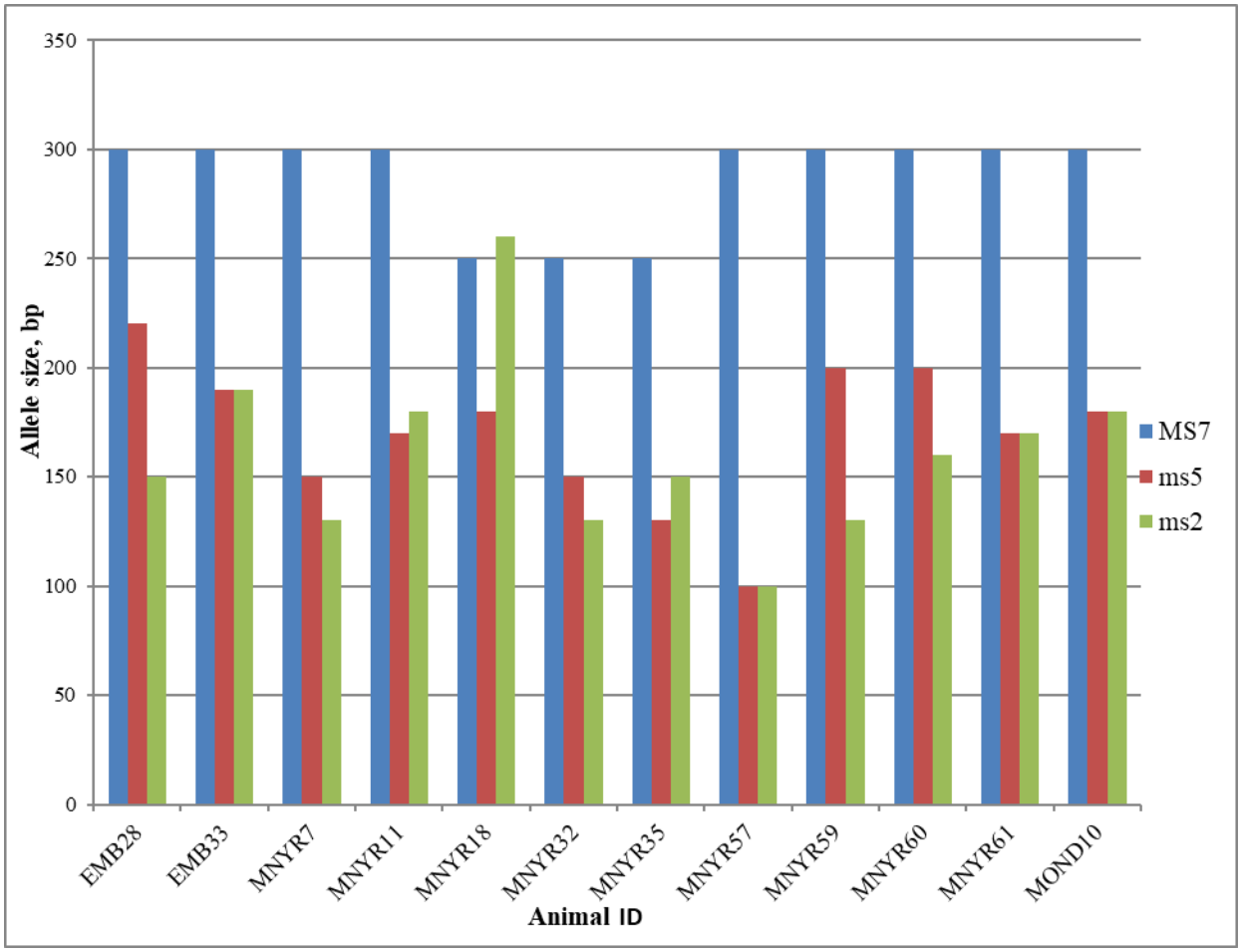

Figure 7: Distribution of all three vaccine markers across cattle groups

4.1.5 *Theileria parva* diversity in different cattle groups

Comparison of the allelic polymorphisms at the 3 satellite loci typed in the present study is shown in fig 8a, 8b and 8c. Parasite diversity across cattle populations was derived by GenAlex where it was determined by the mean number of alleles, and expected heterozygosity. The average expected heterozygosity for MS 7 marker within each of the five populations was highest in Manyara ranch (0.694) and lowest in Esilalei (0.375) with the number of different alleles ranging from 5 (Manyara Ranch) to 2 (Tanga Leila farm, Esilalei and Mswakini) similarly Manyara ranch had the highest number of effective alleles (3.269) and least number of effective alleles was documented in Esilalei (1.6). Likewise, for the ms 5 marker, Manyara ranch revealed the highest parameters, with the mean number of different alleles ranging from 7 in Mswakini to 1 in Loiborsoit. Mean number of effective alleles ranged from 6.321 (Manyara ranch) to 1 (Loiborsoit). Expected heterozygosity was highest in Manyara ranch (0.842) and was least (0.0) in Loiborsoit. Results of the ms 2 marker were not different from those obtained with the 2 other markers; the mean number of ms 2 alleles ranged from to 7 (Manyara ranch) to 1 (Leila Farm), mean number of effective alleles ranged from 5.556 (Manyara ranch) to 1 (Leila Farm). Manyara ranch group had the highest expected heterozygosity (0.82).

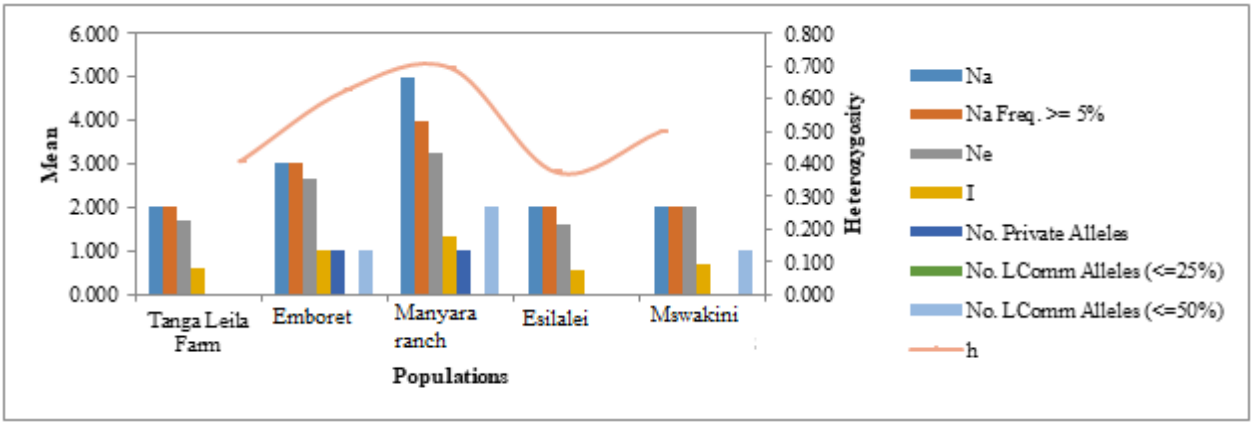

Figure 8a: The mean allelic patterns for MS 7 across the study locations

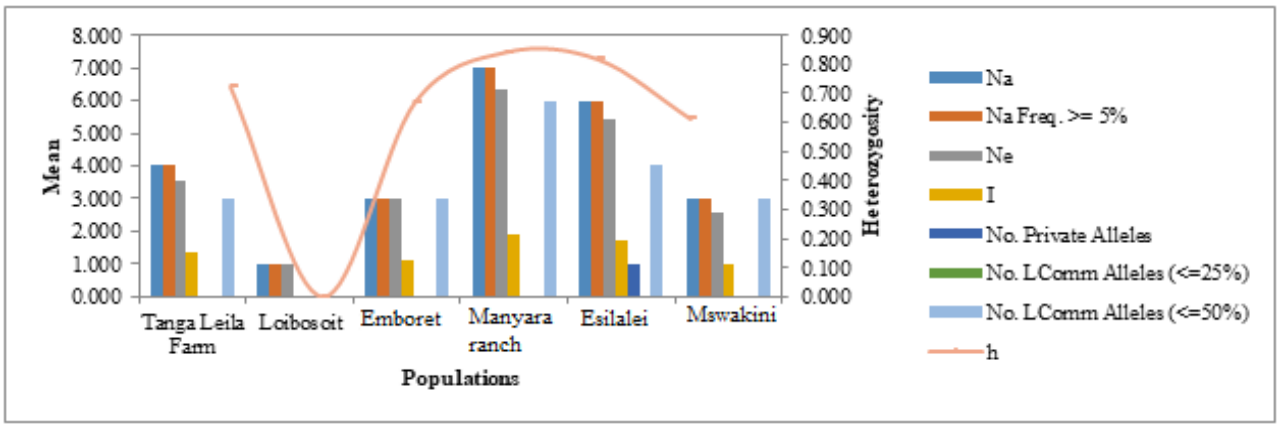

Figure 8b: The mean allelic patterns for ms 5 across study locations

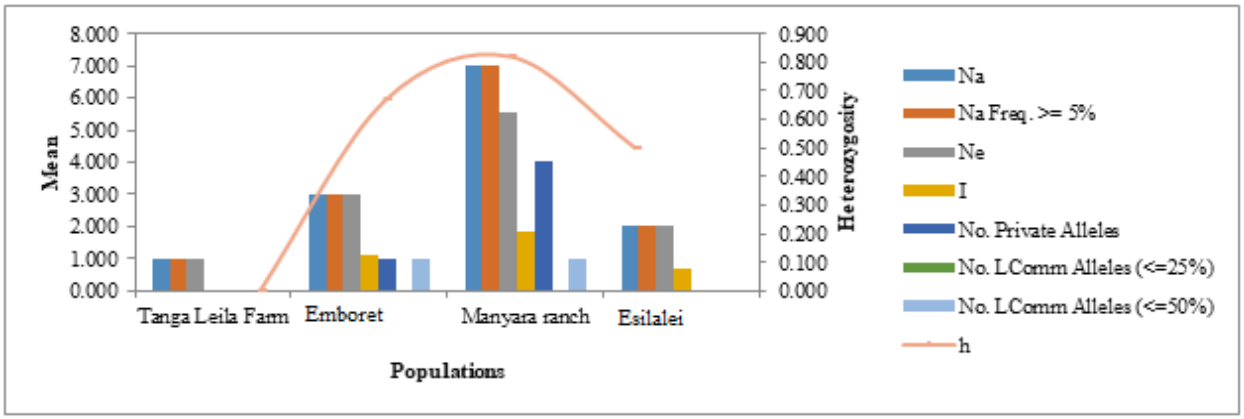

Figure 8c: The mean allelic patterns for ms 2 across the study locations

Na = No. of Different Alleles

Ne = No. of Effective Alleles

1 / (Sum pi^2)

I = Shannon's Information Index

h = Diversity = 1 - Sum pi^2

4.1.6 Principal component analysis and analysis of molecular variance

The Principle Component Analysis (PCA) was constructed to show the relationship between *T. parva* populations from the six cattle groups as shown in (Fig. 9) the results showed patterns of clustering such that most of the parasite alleles (genotypes) were clustered throughout the four quadrants. Most of the Manyara ranch alleles clustered at the top left quadrant together with Esilalei, Emboret and Leila farm alleles. Alleles from

Mswakini were clustered at the top right quadrant together with Esilalei, Manyara Ranch and Loiborsoit alleles. Mswakini alleles were also found at the bottom left quadrant with Emboret and Manyara ranch. The bottom right quadrant contained genotypes from Esilalei, Emboret and Manyara ranch.

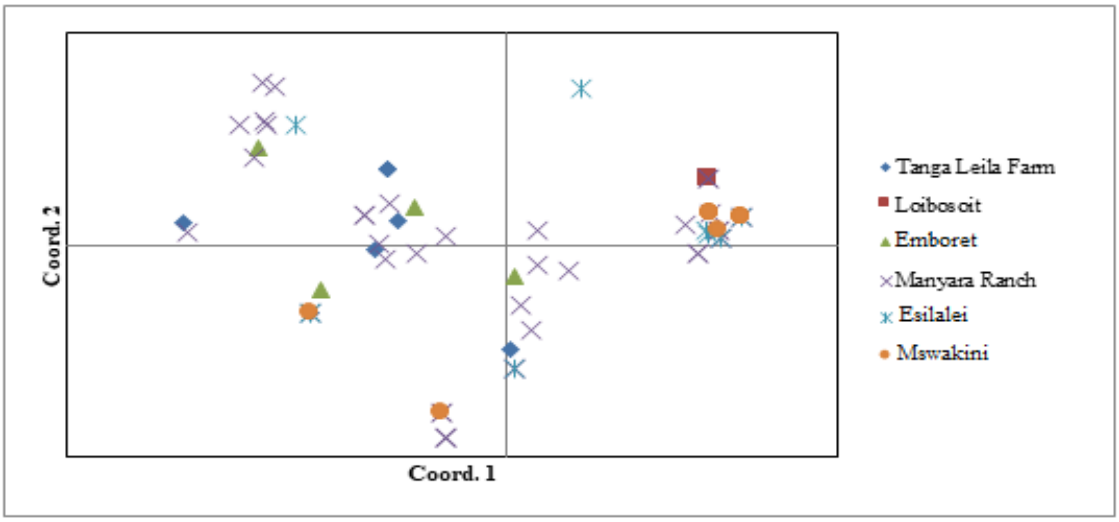

Figure 9: Principal Component Analysis (PCA) of *T. parva* as generated from 6 populations

Analysis of molecular variance (AMOVA) was used to assess the population structure of *Theileria parva* in the six cattle groups by partitioning variation among and within parasite populations. Most of the genetic variation (93%) was contained within populations with only 7% variation between populations.

Table 7: The summary of AMOVA table of the cattle groups

Source	Df	SS	MS	Est. Var	%
Among pops	4	1.587	0.397	0.009	7
Within pops	39	13.299	0.341	0.341	93
Total	43	14.886		0.350	100

4.2 Discussion

The present study revealed an overall prevalence of *T. parva* carrier state to be 34.5%. This value was close to a 37.1% prevalence found by Kazungu *et al.* (2015). While this study sampled cattle from Simanjiro and Manyara districts in the Maasai steppe and farms in eastern Tanzania the study by Kazungu *et al.* (2015) sampled cattle from Simanjiro district of the Maasai steppe and farms in Mwanza, Lake Zone of Tanzania. Hence the difference

in *T. parva* prevalence between the two studies is likely due to different tick control regimes in the different areas. The prevalence of *T. parva* established in cattle in this study was higher than a 31.6% prevalence found by Kimaro, Mor, Gwakisa and Toribio (2017) which was done in Monduli district in the Maasai steppe ecosystem during the dry season. These differences in prevalence may be explained by the ecological and other associated conditions which may favor ticks in different areas where cattle were sampled. The study done by Kimaro *et al.* (2017) sampled cattle during the dry season which is characterized with low tick intensity compared to the wet season during which the present study was conducted. The prevalence values reported in these studies point to a high possibility that most of the animals sampled were in a *T. parva* carrier state, which is a common phenomenon following natural infection or ITM in cattle (Oura *et al.,* 2007; Oura *et al.,* 2011).

The cattle groups in this study were sampled from divergent ecological conditions and management regimes for controlling ticks and tick-borne diseases. It was of interest therefore to compare the cattle groups not only in terms of prevalence of *T. parva* but also how the prevalence varies within and between groups, when the cattle are categorized with respect to whether they were ECF vaccinated or not, longevity of *T. parva* carrier state following ECF vaccination and proximity of cattle grazing areas to wildlife interface areas. Overall a higher *T. parva* prevalence was clearly shown among cattle that were ECF-vaccinated (43%) compared to unvaccinated cattle (13.4%). This finding is in full agreement with the expected norm that ECF vaccination using the live trivalent Muguga cocktail increases the carrier state of *T. parva* among cattle. Likewise, a higher *T. parva* prevalence was found among cattle which grazed in close proximity to the wildlife interface. The higher prevalence (38%) of *T. parva* infection among cattle grazing close to the wildlife interface areas may be explained by the fact cattle that share grazing areas with wildlife reservoirs are likely to be under higher exposure to disease vectors and hence higher *T. parva* positivity.

In order to get a better understanding of longevity or persistence of carrier state following ITM under prevailing conditions in the study areas, this study classified all ECF vaccinated cattle based on their *T. parva* positivity. All the 43% (103/239) *T. parva* positive ECF-vaccinated cattle were further clustered into categories of longevity (duration) since ECF vaccination. The data showed that carrier state was detected in cattle

vaccinated as far as 11 years ago. Comparison of categories of longevity since vaccination revealed that highest frequency of carriers was at 12 months post vaccination these results further confirm previous reports by Oura *et al.* (2007) who detected the carrier state of *T. parva* only up to 4 years. The results reported here have taken advantage of deployment of ITM in the Maasai steppe for at least two decades, and therefore the availability of older cattle vaccinated up to 14 years earlier has provided a useful resource to investigate ITM outcomes in ECF endemic areas.

An interesting question arising from this study was whether the carrier state can be differentiated between ECF vaccinated cattle and those that may have recovered from natural ECF infection. Three VNTR markers, which are constituent components of the Muguga cocktail, were used to monitor carrier state in *T. parva* positive ECF unvaccinated (n=13) cattle. It emerged that 76.9% of this group carried the Muguga vaccine markers (MS 7 and ms 5) indicating that vaccine strains are transmissible to the unvaccinated cattle. Since majority of these cattle belonged to one farm (Leila farm) it may likely be that this is a result of management conditions supporting the transmission of vaccine strains from vaccinated to unvaccinated cattle. This study showed that two of the three vaccine markers (MS 7 and ms 5) were detected in unvaccinated cattle. Although the ms 2 marker was not detected it is uncertain whether this was due to small sample size used in the present work or rather, this study further supports previous findings by Oura *et al.* (2007) where he showed evidence of transmission of some and not all Muguga vaccine components.

The implication of long-term deployment of the ITM, as is the case in northern Tanzania, has not been investigated. Prior to this study, it was hypothesized that long term application of ITM may influence parasite biology, alter genetic diversity of local strains and hence impact on disease dynamics in endemic areas. Although ITM has been employed to protect cattle against ECF in the Maasai herds since 1990s (Di Giulio *et al.*, 2009) no study has preceded this one to investigate *T. parva* genetic diversity in Tanzania. Genotyping of the *T. parva* positive samples using the three VNTR markers allowed to unravel the genetic diversity of *T. parva* in cattle from different locations. The extent of polymorphism varied between the three markers, whereas ms 5 was most polymorphic and MS 7 was least.

Higher genetic diversity was observed among ECF vaccinated cattle compared to unvaccinated cattle. Thus, the 24 alleles across the three markers were all detected with varying frequencies among vaccinated cattle, but only 4 of these were found in

unvaccinated cattle. It is probable that vaccinated cattle would harbor more alleles due to recombination of the local strains with vaccine strains; another explanation for this finding may be due to the ability of the carrier cattle acquiring infection with additional parasite genotypes following tick challenge, thus resulting in carriage of mixed genotypes. This can be used to support the finding that the Manyara ranch group, found at the ecological epicenter of the wildlife interface within the Maasai steppe had the highest mean number of different alleles, effective alleles, expected heterozygosity and private alleles.

While majority of the alleles were shared by individuals from different groups, this study found seven private alleles, each of which was found only in one or the other group. Interestingly all of the private alleles were detected only in vaccinated cattle grazing close to the wildlife interface. The actual mechanisms supporting existence of private alleles are not fully understood but these findings may be ascribed to the notion that the Muguga vaccine is tri-valent, with a multitude of parasite genotypes (Oura *et al.*, 2007; Patel *et al.*, 2011).

In order to gain insight in the population diversity of *T. parva* circulating in the different cattle groups the principle component analysis (PCA) was used. Results revealed different clustering patterns, with most of the alleles clustering together throughout all the four quadrants. The PCA findings strongly suggest the *T. parva* parasites homogeneity among the cattle groups and the absence of a clear association between population genetic structure and the geographical origin of the isolates. Furthermore, analysis of molecular variance revealed higher genetic variations within individual isolates (97%) compared to within *T. parva* populations (3%). One possible explanation to this finding is the occurrence of a high rate of crossing between different *T. parva* isolates and recombination within the parasite population hence the lower diversity within the *T. parva* populations. This was somehow surprising considering that cattle groups sampled from Tanga (eastern Tanzania) were expected to carry distinct satellite alleles, separate from thosefound in cattle from the Maasai steppe (northern Tanzania). It is possible that uncontrolled cattle movements between the different regions in Tanzania may have contributed to the similarity of the parasite genotypes.

The use of ITM in Tanzania has been increasing gradually since 1998 (Di Giulio *et al.*, 2009; Martins *et al.*, 2010). This served as one of the motives for the present study in order to understand implications of the long term deployment of ITM for over 20

years in the Maasai areas, Thus the study has brought forth clear findings showing that the carrier state induced by Muguga cocktail vaccine is effective and it induces a long lasting immunity detectable up to 11 years post vaccination something which hitherto has not been reported. Furthermore, results of this study have allowed to deduce the questionable viewpoint on the spreading of the vaccine strains to the unvaccinated cattle via tick vectors. In this study, no ticks were investigated, notwithstanding it was clearly demonstrated that two of the three vaccine markers were detected in several co-grazing unvaccinated cattle. These findings corroborate previous reports by Olds *et al*. (2018).

A significant finding emanating from this study is that vaccination against ECF has an influence on the diversity of *T. parva* parasites, whereby greater number of alleles were shown in the vaccinated cattle compared to the unvaccinated cattle. It was speculated that the enhancement of diversity is a direct outcome of the live vaccination process in the field, whereby the vaccine strains may potentially recombine with local strains to generate more genotypes. It may be speculated that wider *T. parva* diversity plays a significant role to restrict breakthrough infections in the vaccinated cattle, as was observed during the conduct of this study, whereby no clinical ECF cases were encountered among sampled cattle. The study took into consideration separate ecological and geographical locations from where the samples were collected. Interestingly, analysis of *T. parva* populations revealed that geographical separation did not necessarily imply differences in the genetic structure of *T. parva* populations.

Summing up, majority of the cattle investigated in this study were sampled from wildlife interface areas. Such areas support constant interaction between cattle, wildlife reservoirs, tick vectors and the parasites. Ecological pressure in such an interface presumably drive the establishment of a carrier state in cattle differently as it would happen in cattle populations grazed far from wildlife. Therefore, the role of the wildlife interface on the diversity of *T. parva* may not be negated, as highest parasite diversity shown in this study was among ECF vaccinated cattle found in close proximity to wildlife interface.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This study was conducted in seven different locations to study the prevalence, persistence and diversity of *T. parva* in cattle vaccinated against East Coast Fever using the p104 gene and three different VNTR markers (Minisatellite 7, microsatellite 2 and 5). The results suggest that ITM can indeed provide a lifelong immunity since there was vaccine markers detection in the vaccinated cattle up to 11 years post vaccination. Vaccinated cattle and the ones grazing close to the wildlife interface had higher prevalence of the carrier state. There was detection of vaccine markers in the unvaccinated cattle which shows the role of ticks in transmission of *T. parva* parasites among the co grazing animals. Vaccination against ECF has an influence of *T. parva* diversity as a greater number of alleles was detected in the vaccinated cattle compared to the co grazing unvaccinated cattle. Wildlife interface areas and uncontrolled cattle movement increases the *T. parva* diversity as the highest diversity was from areas close to the wildlife. Moreover, there was a similarity in the circulating *T. parva* strains among the different geographical locations used in this study, as there was sharing of some alleles among the different cattle populations. The carrier state was observed in only 43% of the vaccinated cattle and it decreases with time due to clearance of the parasites by the cattle adaptive immunity. However no breakthrough infections were observed throughout the study therefore it cannot be concluded whether the ITM vaccination should be done routinely or just once.

5.2 Recommendations

Basing on the finding that ITM causes a long life immunity detected up to 11 years post vaccination and evidence of recombination between vaccine strains and local *T. parva* strains and the higher parasite diversity established in the cattle grazing close to the wildlife interface areas and in vaccinate cattle I strongly recommend the following;

(i) Pastoralists to focus more on the use of ITM to prevent ECF due to the protective nature of the carrier state induced by it and the long-lasting immunity provided.

(ii) Pastoralists should vaccinate all of their cattle not just a portion of them as it is a common practice among them, this contributed to the lower prevalence of carrier state in the unvaccinated cattle. The cause of this maybe be due to the costly nature of the Muguga cocktail vaccine costing from 6 dollars to 10 dollars per animal

depending on the animal's size making it hard for them to afford it. Although natural infection can also cause the carrier state but it may not be as effective and long lasting as the one caused by ITM. Vaccination of a larger proportion or all of their herds would increase the protective effect resulted from the carrier state.

(iii) Co-grazing of the vaccinated and unvaccinated cattle should also be avoided as this contributes to the diversity of the *T. parva* strains due to the recombination of the vaccine derived and local strains. Therefore, pastoralists should separate their vaccinated cattle from the unvaccinated ones

(iv) Pastoralists should control the cattle movement and avoid grazing of cattle near to the wildlife interface areas as these areas have shown to increases the diversity of the *T. parva*. Therefore, they should avoid the interaction of the cattle with the buffalos which are the main reservoirs of *T. parva* harboring more divergent strains of *T. parva*.

Further studies should be done on ticks to identify whether the *T. parva* strains circulating in them are similar to the ones found in the cattle

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APPENDIX

Appendix: showing some of the data used to generate the results of this study

ID no	ECF vaccination status	Proximity to wildlife	p104	MS 7	ms 5	ms 2
EMB1	1	1	1	0	0	0
EMB28	1	1	1	300	220	150
EMB32	1	1	1	450	0	180
EMB33	1	1	1	450	190	190
EMB35	1	1	1	350	100	0
LB18	2	1	1	0	200	0
LB22	1	1	1	0	0	0
LB24	1	1	1	0	0	0
LB34	1	1	1	0	0	0
LB8	2	1	1	0	200	0
MNYR1	1	1	1	150	150	0
MNYR10	1	1	1	0	0	0
MNYR11	1	1	1	300	170	180
MNYR12	1	1	1	0	0	0
MNYR13	1	1	1	400	170	0
MNYR14	1	1	1	0	0	0
MNYR15	1	1	1	0	160	0
MNYR16	1	1	1	300	0	0
MNYR17	1	1	1	300	150	0
MNYR18	1	1	1	250	180	260
MNYR19	1	1	1	150	0	0
MNYR2	1	1	1	300	100	0
MNYR20	1	1	1	0	130	0
MNYR21	1	1	1	300	170	0
MNYR23	1	1	1	150	0	0
MNYR24	1	1	1	150	0	0
MNYR25	1	1	1	0	180	0
MNYR26	1	1	1	0	0	0
MNYR27	1	1	1	0	130	0
MNYR28	1	1	1	0	0	0
MNYR3	1	1	1	0	200	0
MNYR30	1	1	1	250	0	0
MNYR32	1	1	1	250	150	130
MNYR33	1	1	1	0	0	0
MNYR34	1	1	1	0	0	0
MNYR35	1	1	1	250	130	150
MNYR36	1	1	1	250	130	0
MNYR37	1	1	1	250	170	0
MNYR38	1	1	1	0	0	0
MNYR4	1	1	1	300	100	0